ISSN 0006-3509, Biophysics, 2010, Vol. 55, No. 3, pp. 467–472. © Pleiades Publishing, Inc., 2010.
Original Russian Text © T.V. Machneva, N.N. Bulgakova, Yu.A. Vladimirov, A.N. Osipov, 2010, published in Biofizika, 2010, Vol. 55, No. 3, pp. 532–538.

COMPLEX SYSTEMS BIOPHYSICS

The Role of Endogenous Porphyrins in Laser Therapy of Experimental Skin Wounds

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Received February 15, 2010

Abstract—The role of endogenous porphyrins in the effect of laser irradiation on the superoxide dismutase (SOD) activity of wound exudate and rat leukocyte activity has been studied on models of aseptic incised skin wounds. Wounds were irradiated with a He–Ne laser (632.8 nm, 1.5 J/cm^2) on the 2nd, 3rd, and 4th days after the beginning of the experiment. Irradiation effects were evaluated by the SOD activity (NBT test) and the activity of leukocytes of the wound exudate (as a chemiluminescent response to opsonized zymosan). It was found that in animals subjected to laser irradiation, the SOD activity sharply increased. This effect depended on endogenous porphyrin concentration and was retained throughout the experiment. The SOD activity in unirradiated animals decreased from the 2nd to the 5th day of experiment. The evaluation of the activity of wound exudate leukocytes did not reveal any distinct dependence of the effect on the concentration of endogenous porphyrins.

Key words: skin wounds, laser irradiation, wound exudate, superoxide dismutase activity, functional activity of leukocytes

DOI: 10.1134/S000635091003019X

INTRODUCTION

Low-intensity laser radiation is applied in modern medicine for prevention and treatment of a number of pathological conditions [1–6], including faster wound healing [7, 8]. However, dose adjustment and the choice of radiation type are mainly done in the empirical way, and the result of laser therapy does not always prove positive [4–6]. To a large extent this is connected with that the primary acceptor of laser radiation in cells in tissues has not yet been established. In the contemporary literature sources, several substances are considered in the capacity of possible acceptors: porphyrins, dinitrosyl complexes of hemoglobin, superoxide dismutase (SOD) and some others [9–12]. In 1994 a hypothesis was formulated on three main mechanisms of action of low-intensity laser radiation [12]. According to one of them, laser radiation causes a photodynamic action, in particular, on phagocyte membranes, which is accompanied by an increase of their permeability and as a result, growth of the concentration of intracellular calcium, which is what eventually leads to phagocyte activation. Primary acceptors of laser radiation in this case may be presented by endogenous porphyrins [10, 11]. Endogenous porphyrins are present in blood plasma, their amount increases in various pathological conditions (including the wound process) [13, 14].

In the present work we studied the role of endogenous porphyrins in the action of optical radiation in the red region of the spectrum on the functional activity of leukocytes and SOD activity of the wound exudate in rats.

EXPERIMENTAL

Use was made of standard Hanks' solution (Institute of Polyomyelitis and Viral Encephalitides, Russia), β-NADH (Applichem, Denmark), 5-methyl phenazine methosulfate (Fluka, Switzerland), NitroBlue Tetrazolium (NBT; Dia-M, Russia), DMSO and diethyl ether (Khimmed, Russia), zymosan and luminol (Serva, Germany); other reagents were domestic products of special, chemical, and analytic purity grades.

Experiments were performed on albino male rats (RPC Gidrobios, RF) of body mass 130–160 g, kept under standard vivarium conditions one animal per cage and receiving complex pelleted laboratory feed with constant access to water. Measurement of SOD activity of the exudate and functional activity of leukocytes was conducted on two groups of animals: 1st group (control) without irradiation($n = 3$); 2nd group with laser irradiation of wounds ($n = 7$ in studying

Abbreviations: CL, chemiluminescence; SOD, superoxide dismutase; PP, protoporphyrin.

Editor's Note: I certify that this text exactly reproduces all factual statements (just correcting some chemical names) and closely conveys the phrasing and style of the original publication. *A.G.*

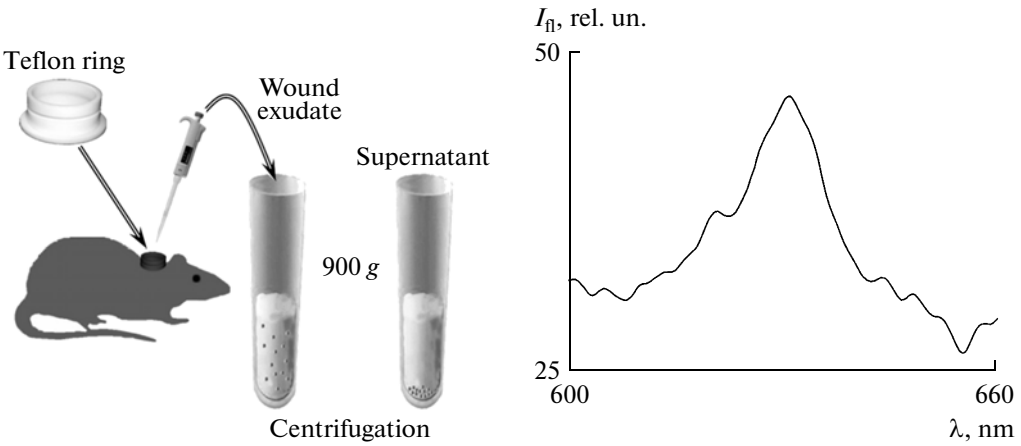

Fig. 1. The method of conducting experiments. Creation of a model wound with the aid of a Teflon ring; collection of the wound exudate from the wound surface; sedimentation of cells by centrifugation and measurement of fluorescence of endogenous porphyrins in the supernatant. Wavelength of fluorescence excitation – 406 nm.

SOD activity of wound exudate, $n = 6$ in studying the functional activity of the leukocytes of wound exudate).

Experimental wound model, sampling of wound exudate and leukocyte isolation. All experiments were performed on the model of a full-thickness plane skin wound proposed by Slutskii [15]. Specific features of wound creation and exudate collection have been described by us earlier [16, 17].

The wound exudate was collected with automatic pipettes into plastic tubes (Fig. 1) in the beginning of the experimental day before irradiation from the second to the fifth day after wounding. From the wound exudate we isolated polymorphonuclear leukocytes for determination of chemiluminescence (CL) and the supernatant for determination of SOD activity and registration of endogenous porphyrin fluorescence. Cells were isolated by our modification of the method described in [18]. The detailed procedure of leukocyte isolation was described earlier [17]. All cells were kept in the cold and used within 4 h after exudate collection.

Fluorescence of endogenous porphyrins in rat wound exudates was measured with the aid of an optical spectrometer FS-003V (LLC Klaster, Russia) permitting remote measurement of optical spectra from the surface or volume of various materials, including spectra of reflection and fluorescence of biological objects. The spectrometer comprised a grid polychromator with a multichannel photoreceiver united into a unified module with laser sources of excitation. For delivery of the excitatory laser radiation to the specimen examined and of the registered fluorescence radiation to the photoreceiver, we employed a Y-shaped ring fiber-optic catheter (7 fibers of 100 μm diameter with numerical aperture 0.22). The operative range of the spectrometer was 410–1000 nm, the spectral resolution with the use of the fiber-optic catheter was 3 nm. For fluorescence excitation use was made of the radiation of a diode laser with a wavelength of 407 nm, which falls on the Soret band in the absorption spectrum of porphyrins [14, 19]. The exposure time was 10 ms. The power of the excitatory laser radiation from the end of the catheter was 3 mW.

The fluorescence of endogenous porphyrins was registered in the supernatant obtained after centrifugation of the wound exudate (Fig. 1). In measurement of the fluorescence of wound exudates in the presence of an exogenous photosensitizer, the sample was supplemented with protophorphyrin (PP) IX (as a solution in phosphate buffer with Triton X-100) in a volume not exceeding 10% of the total. The total sample volume in most cases was 150 μL, but occasionally it was smaller (down to 50 μL) because of the limited amount of exudate obtained from some rats.

Wound irradiation was performed with the aid of a He–Ne laser (632.8 nm) ULF-01 (RF) on the second, third, and fourth day after inflicting the wound immediately after sampling the wound exudate. The procedure and irradiation dose calculation have been described by us earlier [16].

Measurement of luminol-dependent CL of wound exudate leukocytes was conducted in accordance with the standard procedure described in works [16, 17]. The sample volume was 1 mL, the number of cells in the sample was 150 thousand. The sample included luminol (4×10^{-7} M).

Addition of zymosan (0.8 mg/mL) led to leukocyte activation, after which activated CL was registered. CL intensity was expressed in rel. un. characterizing the difference between the maximal level of activated CL and the level of spontaneous CL (without zymosan). The functional activity was estimated by the chemiluminescent response in the form of ratio I_n/I_2, where I_n is CL intensity on the 3rd, 4th or 5th day after wounding, I_2 is that on the 2nd day.

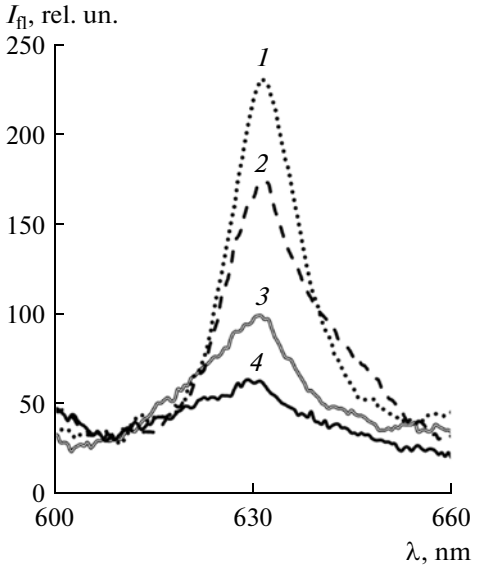

Fig. 2. Typical fluorescence spectra of wound exudates without and in the presence of endogenous porphyrin at an excitation wavelength of 410 nm. The wavelength of fluorescence maximum – 632 nm; *1* – protoporphyrin IX added into the wound exudate of rat no. 4 to a final concentration of 50 nM; *2*, *3* and *4* – wound exudates of rats with nos. 5, 4 and 1 respectively.

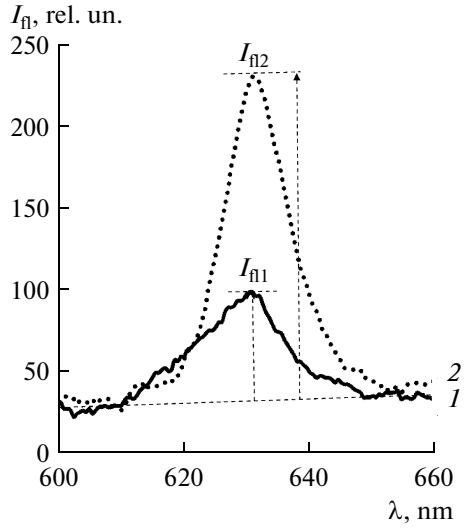

Fig. 3. Fluorescence spectra of the wound exudate of rat no. 4 without (*1*) and in the presence (*2*) of endogenous porphyrin. Protoporphyrin IX added into the wound exudate of rat no. 4 to a final concentration of 50 nM. I_{fl1} and I_{fl2} are the maxima of the peaks of fluorescence spectra of the sample before and after addition of the standard porphyrin solution. The concentration of endogenous porphyrins in the corresponding wound exudate constituted 29.92 nM.

Measurement of SOD activity of wound exudate was conducted by the standard method with NBT [20]. For generation of superoxide radical we used the reaction of oxidation of reduced β-NADH by 5-methyl phenazine methosulfate [16]. The results were expressed as the ratio A_n/A_2, where A_n is SOD activity on the 3rd, 4th or 5th day after wounding, A_2 is that on the 2nd day.

RESULTS AND DISCUSSION

In the first series of experiments the conditions were selected for registration of the fluorescence of endogenous porphyrins in wound exudates. It is known that the wound exudate in its biochemical composition is close to the blood plasma [15, 21]. Since endogenous porphyrins are present in blood and plasma [11, 13, 14], one should expect their detection in the wound exudate as well. Typical fluorescence spectra of wound exudates are presented in Fig. 2 (curves *2*–*4*). One can see that they contain peaks of fluorescence in the red range with maxima in the region of 632 nm, which can be ascribed to endogenous porphyrins. To confirm this suggestion under analogous conditions of excitation we measured the fluorescence spectra of PP IX, which was added into rat wound exudates at different concentrations. Presented in Fig. 2 (curve *1*) is one of the spectra obtained in this way. One can see that the fluorescence maxima of exogenous PP IX and endogenous porphyrins are positioned very closely. This confirms the suggestion about fluorescence of namely the endogenous PP IX under the given conditions of experiment. As it is seen in Fig. 2, the intensity of fluorescence peaks in the region of 632 nm differed for different rats (curves *2*–*4*), which testifies to a different content of endogenous porphyrins.

For quantitative determination of the content of endogenous porphyrins in the exudate after measuring the fluorescence it was supplemented with a standard solution of PP IX and the fluorescence was measured again. An example of such an experiment is presented in Fig. 3. Calculation was performed with the use of equation:

$$C_x = C_s \frac{I_{fl1}}{I_{fl2} - I_{fl1}},$$

where I_{fl1} and I_{fl2} are the peaks of fluorescence spectra before and after addition of the standard porphyrin solution, while C_x and C_s are the porphyrin concentration in the initial sample and the final concentration of the added standard solution of PP IX respectively. The endogenous protoporphyrins in the wound exudate will further be called endogenous porphyrins.

As a result of the conducted measurements in the exudates of all examined animals we have determined the concentrations of endogenous porphyrins (table). In different rats these values varied from 21.69 to 133.96 nM. It was of interest to elucidate how these differences influence the sensitivity of wounds to laser irradiation.

Content of endogenous porphyrins (C, nM) in the wound exudate of rats ($n = 11$) determined with the aid of the fluorescent method

Rat no. in experiment	C, nM	Standard deviation
1	23.9	0.4
2	25.6	4.4
3	27.4	4.5
4	10.6	0.6
5	12.9	2.2
6	15.0	0.2
7	29.9	3.9
8	31.3	3.0
9	34.1	5.0
10	14.0	4.8

Note: Rats nos. 1–8 – test (subjected to laser irradiation), nos. 9–11 – control (without irradiation).

We have earlier shown that laser radiation can exert substantial influence on the SOD activity of the rat wound exudate [16]. At that one and the same dose could lead to different effects, which, as we suggested, was conditioned by the different concentration of endogenous photosensitizers. It appeared of interest to investigate the influence of various concentrations of endogenous porphyrins on the wound process under conditions of laser irradiation. It is known that the first stage in wound healing is inflammatory [15, 21], and it is in the inflammatory process that antioxidant protection is especially important [20]. Figure 4 presents the results of investigation of the laser radiation-induced change of the SOD activity of rat wound exudates.

The SOD activity of cells was estimated in the form of the ratio A_n/A_2, where A_n is SOD activity on the third (Fig. 4a), fourth (Fig. 4b) or fifth (Fig. 4c) day after making the wound, A_2 – on the second day. Obviously, the change of SOD activity had a similar dependence on the content of endogenous porphyrins in the course of three days of observation of the wound process. For all irradiated animals (test group) we observed an increase of the SOD activity of the exudate (Fig. 4, light circles) as compared with the second day of the wound process, which is confirmed also by the results obtained by us earlier [16, 22]. On average the SOD activity in the test group increased fivefold by the fifth day of the wound process (Fig. 4c).

An especially strong influence of laser radiation on the parameter investigated manifested itself in the interval of porphyrin concentrations from 13 to 34 nM. The maximal effect was observed on the fifth day—SOD activity increased 8.5 times at a porphyrin content equal to 29.9 nM (Fig. 4c).

In rats not subjected to laser irradiation (Fig. 4, dark circles) the SOD ay insignificantly but gradually decreased. Therewith the porphyrin concentration in these rats was in the interval (13–34 nM) in which for rats of the test group we observed maximal effects. On average the SOD activity of the wound exudate in the control group by the fifth day decreased 3.8 times relative to the second day of the wound process. It is necessary to note that earlier we have more than once shown an analogous decrease of SOD activity of wound exudates of rat not subjected to the action of laser radiation [16, 22].

As a result of the conducted series of experiments it became obvious that laser light exerts a significant effect on the ability of the wound exudate to intercept superoxide anion radicals. On the basis of the results obtained we can make a conclusion that during the action of radiation in the red region of the spectrum on the SOD activity of the rat wound exudate the endogenous photosensitizers can be porphyrins, the concentration of which is what determines the effects of laser radiation.

It is known that activation of leukocytes is directly connected with a change in the production of reactive oxygen species, in particular superoxide anion radical [12].

We have earlier shown that laser radiation exerts effective influence on the functional activity of leukocytes of rat wound exudates [16, 17] and leukocytes of human blood [10, 11]. Different doses of irradiation led to alternately directed effects, which we, just as in the case with SOD activity, associate with the different amount of endogenous photosensitizers. Indeed, in conducting investigations on isolated human blood leukocytes we have shown a dependence of the laser radiation-induced change of cell activity on the level of endogenous porphyrins [10, 11].

In the given series of experiments it was interesting to elucidate the role of porphyrins in the action of laser radiation in the red region of the spectrum on the leukocytes of the wound exudate. A study of their influence on these cells can become an important link in the therapy of the wound process, because they make a substantial contribution to wound healing at the stage of inflammation [21].

The functional activity of cells was evaluated by the change of the chemiluminescent response (Fig. 5) in the form of ratio I_n/I_2, where I_2 is CL intensity of wound exudate leukocytes on the second day after making the wound, while I_n is on the third (Fig. 5a), fourth (Fig. 5b) or fifth (Fig. 5c) day respectively.

Just as in the investigations conducted earlier [16, 17], in the majority of animals of the test group laser radiation led to restitution or increase of the wound exudate leukocyte activity (Fig. 5, light circles), while in the control group the cell activity as compared with the second day of the wound process declined (Fig. 5, dark circles).

From the data obtained (Fig. 5, light circles) it is evident that a substantial influence of the concentra-

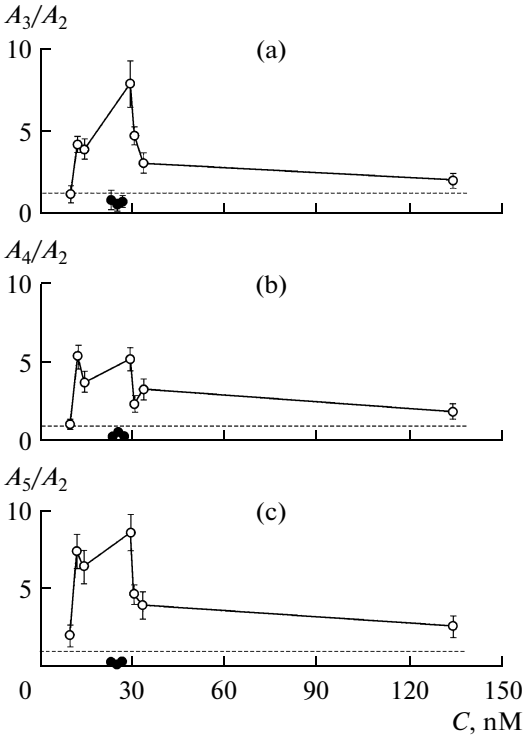

Fig. 4. Change in the relative SOD activity (A_n/A_2) of the exudate on the third (a), fourth (b) and fifth (c) day of the course of the wound process as compared with SOD activity on the second day (A_2) after making the wound as dependent on the concentration of endogenous porphyrins (C, nM) upon laser irradiation of the sample; light circles, irradiated animals; dark circles, nonirradiated animals (control).

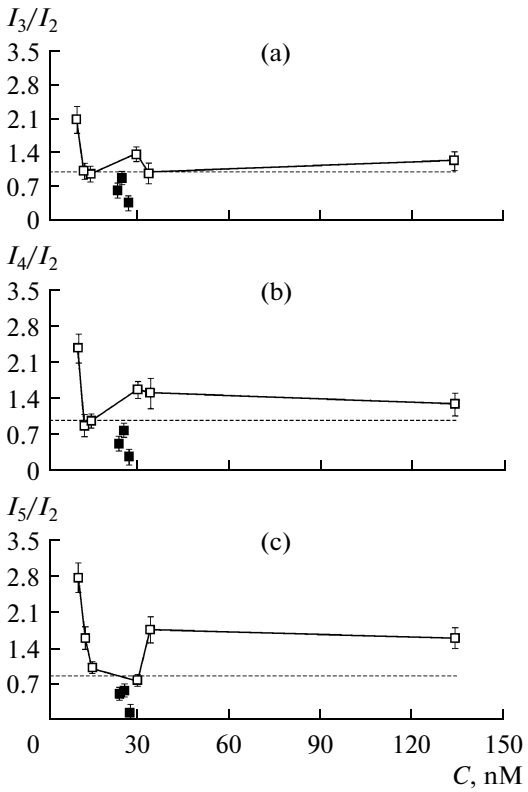

Fig. 5. Change in the functional activity of leukocytes determined by the ratio of CL intensity I_n/I_2 (where I_n is CL intensity on 3–5 days after making the wound, I_2 – CL intensity on the second day) as dependent on the concentration of endogenous porphyrins (C, nM) upon laser irradiation of the exudate: (a) $n = 3$; (b) $n = 4$; (c) $n = 5$; light circles, irradiated animals; dark circles, nonirradiated animals (control).

tion of endogenous porphyrins on the chemiluminescent response is not attained. However in the concentration interval from 13 to 34 nM some decline takes place in the laser radiation-induced growth of the chemiluminescent response, giving place to a raise, which is especially pronounced on the fifth day of the wound process (Fig. 5c).

Earlier in conducting investigations in vitro on isolated human blood leukocytes we have shown a dependence of the laser radiation-induced change of cell activity on the level of endogenous porphyrins [10, 11]. However in the given investigation the irradiation was conducted in vivo and leukocytes were isolated from the wound exudate of rats. Probably, in these conditions a role is played by other mechanisms of laser-induced change of cell activity. Possibly, the cause of the absence of a clear-cut dependence on porphyrin concentration consists in that the change in the activity of wound exudate leukocytes after the actin of visible radiation represents not a primary but a secondary process. The parameter under study cam change in response to a series of previous events, for example, to laser-induced increase of wound exudate SOD activity, the more so that the porphyrin concentration interval (13–34 nM) in which significant changes occurred in both SOD activity and chemiluminescent response coincides.

It is possible that in other conditions (for example, upon irradiation of isolated leukocytes of wound exudate in vitro without and in the presence of wound exudate) the endogenous porphyrins may completely determine the cell response to laser irradiation.

ACKNOWLEDGMENTS

We express our gratitude to P.A. Ol'khovskii and D.M. Protopopov for help in executing the given work.

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